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Genome-Scale Model of *Streptococcus thermophilus* LMG18311 for Metabolic Comparison of Lactic Acid Bacteria▽†

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In this report, we describe the amino acid metabolism and amino acid dependency of the dairy bacterium *Streptococcus thermophilus* LMG18311 and compare them with those of two other characterized lactic acid bacteria, *Lactococcus lactis* and *Lactobacillus plantarum*. Through the construction of a genome-scale metabolic model of *S. thermophilus*, the metabolic differences between the three bacteria were visualized by direct projection on a metabolic map. The comparative analysis revealed the minimal amino acid auxotrophy (only histidine and methionine or cysteine) of *S. thermophilus* LMG18311 and the broad variety of volatiles produced from amino acids compared to the other two bacteria. It also revealed the limited number of pyruvate branches, forcing this strain to use the homofermentative metabolism for growth optimization. In addition, some industrially relevant features could be identified in *S. thermophilus*, such as the unique pathway for acetaldehyde (yogurt flavor) production and the absence of a complete pentose phosphate pathway.

Lactic acid bacteria (LAB) are of great importance in the food industry because of their lactic acid production and their characteristic impact (e.g., texture, flavor) on the final product (19). LAB, as fastidious organisms, require a complex medium (such as milk) and are dependent on their proteolytic system for their supply of essential amino acids (34). Amino acids are not only the building blocks for proteins and peptides, but they also serve as precursors for many other biomolecules (1). Amino acids are also important for the final flavor of a product. Most amino acids do not directly influence the product flavor, but they will contribute indirectly to it because they are precursors of aromatic compounds (36). The conversion of amino acids to flavor compounds is initiated mainly by amino acid transamination, which uses an α-keto acid as an amino group acceptor for the aminotransferases (27). The presence (or absence) of the α-keto acid either by endogenous production or by addition to the medium is an important factor in flavor formation (13). The α-keto acids are decarboxylated into aldehydes, which are the precursors of other flavor compounds such as alcohols, esters, and carboxylic acids (27). A large variation in flavor formation between strains and species is observed. Different studies have reported this biodiversity (25, 27, 32, 33); van Hylckama Vlieg et al. studied, for instance, the difference between dairy and nondairy lactococcal strains, since the latter group has some unique flavor-forming activities (33).

Amino acid catabolism and anabolism are complex processes, and thus, metabolic models will be helpful for their understanding. Genome-scale metabolic models provide an overview of all metabolic conversions in an organism based on its genome sequence and make it possible to visualize different metabolic pathways, such as amino acid metabolism. These models can be used to understand the metabolism and can then be applied for a directed study of functionality. For *Lactobacillus plantarum* and *Lactococcus lactis*, such genome-scale models have already been developed (18, 29); the construction of such a model for *Streptococcus thermophilus* LMG18311 is described in this paper. The characterization of the genome sequence of this *S. thermophilus* strain has revealed the presence of a large number of incomplete or truncated genes. These so-called pseudogenes amount to 10% of the total genes, and most of them relate to carbohydrate metabolism, transport, and regulation (2, 11). *S. thermophilus* is an important starter for the dairy industry. It is used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* for the production of yogurt. It is also used for the manufacture of cheeses in which high cooking temperatures are applied (11). The objective of this paper is to study the metabolism of *S. thermophilus* with the use of genome-scale models and experimental data in a comparative way. This comparison with other LAB may reveal important differences. This study showed the simple primary metabolism and the extensive amino acid metabolism of *S. thermophilus*.

MATERIALS AND METHODS

Construction of the genome-scale model. Genome-scale models are based on annotated genome sequences and experimental data and have become available

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for an increasing number of organisms, including various LAB (20, 30). A useful tool for the construction of these in silico models is the SimPheny software package (Genomatica Inc., San Diego, CA). The in silico models are based on a thorough metabolic reconstruction of well-annotated genome sequences (29). The reconstruction of the network of *S. thermophilus* LMG18311 (2, 11) was initiated by an automatic first reconstruction using the autograph method (*au*tomatic *t*ransfer by *o*rthology of *g*ene *r*eaction *a*ssociations for *p*athway *h*euristics) as described in much detail elsewhere (18). The automatic output of the autograph method was subsequently curated extensively to accommodate the available annotation and literature on metabolic pathways and enzymes, a process described in detail elsewhere (8). Also part of the curation was the comparison of the gene reaction associations with the available annotations in KEGG (http://www.genome.jp/kegg/) and in the ERGO bioinformatics suite (http://ergo.integratedgenomics.com/ERGO/) (26).

Bacterial strains, media, and growth conditions. The strains used in this study were *S. thermophilus* LMG18311 (2), *L. lactis* MG1363 (35), and *L. plantarum* WCFS1 (14). Cells were grown anaerobically in chemically defined medium (CDM) (15, 21, 23), containing the amino acids (see Table S5 in the supplemental material), at 42°C, 30°C, and 37°C, respectively.

Amino acid omissions. Cells of *S. thermophilus* were grown overnight in CDM (15) containing all 20 amino acids in the concentrations shown in Table S5 in the supplemental material. The overnight cultures were washed twice at 4°C in a Megafuge 1.0R (Heraeus Instruments, Germany) in phosphate-buffered saline.

CDM without amino acids was freshly prepared for each experiment. Different combinations of amino acids were added to this medium. The amino acids were added in the same concentrations as those used in complete CDM. We started with single omissions of amino acids followed by multiple omissions until we found the most minimal combination. The concentrations of the different amino acids supplied are listed for the different experiments (see Table 2). The different minimal defined media were inoculated 0.5% in triplicate with the washed overnight culture, and growth was followed by measuring the optical density at 600 nm (OD_{600}).

Growth on defined medium (chemostat). Fermentations were performed in duplicate as described by Teusink et al. (30). *S. thermophilus* LMG18311 was grown at 42°C in CDM in a 50-ml tube and used as the inoculum of 500 ml of pH-controlled (pH 6.5) CDM, while the medium was 1% inoculated. Fermentations were performed in a 2-liter fermentor (Applikon Biotechnology BV, The Netherlands). The fermentations were controlled by a Bio Controller ADI 1010 and by a Bio Console ADI 1025 (Applikon Biotechnology BV, The Netherlands). The headspace was flushed with nitrogen (10 ml min^{-1}) at a stirring speed of 100 rpm. At an OD_{600} of ~0.5, the medium pump was switched on to reach a dilution rate of 0.4 h^{-1}. Steady-state conditions were achieved within five volume changes (30). The dilution rate was changed three times, so a total of four dilution rates was achieved (0.1 h^{-1}, 0.2 h^{-1}, 0.3 h^{-1}, and 0.4 h^{-1}). At each steady state, four 50-ml samples were taken and spun down at 4°C in a UniCen MR centrifuge (Herolab, The Netherlands). Supernatant was used for high-performance liquid chromatography (HPLC) analysis of organic compounds (28).

GC analyses. For the identification of volatile components in the samples, purge-and-trap thermal desorption cold-trap gas chromatography (GC) was used as described previously (7, 27). The headspace samples were concentrated on a Fisons MFA 815 cold trap (CE Instruments, Milan, Italy), followed by separation on a GC-8000 top gas chromatograph (CE Instruments) equipped with a CP-Sil 5 CB low-bleed column (Chrompack, Middelburg, The Netherlands) and detection by a flame ionization detector. The GC data were processed in MetAlign, a tool (developed by Plant Research International, The Netherlands) to align spectra and to identify significant differences between the spectra (6, 16).

HPLC analyses. Extracellular metabolites present in the supernatant of fermentation samples were measured using reversed-phase HPLC analysis with a C_{18} column as described elsewhere (28).

RESULTS

Genome-scale model development. A genome-scale metabolic model for *S. thermophilus* has been developed based on the annotated genome of strain LMG18311 (2, 11). The available models of *L. plantarum* (30) and *L. lactis*, which were constructed using the autograph method (18), were used for the construction and development of the *S. thermophilus* model. Based on these models, many gene-protein relationships and non-gene-associated reactions could be incorporated into our model, resulting in a metabolic map of *S. thermophilus* (Fig. 1).

Different features of every gene, such as correct annotation, function, and corresponding EC number, were checked manually before they were included in (or excluded from) the model. Examples of excluded genes are truncated, hypothetical, and nonmetabolic. Excluded genes are not deleted and can be included again later when the function of such a gene has been identified. Genes coding for metabolic enzymes have been included and associated with the corresponding reactions (30). Also, non-gene-associated reactions, based on biochemical and experimental evidence (fermentations, amino acid omissions), were added to close gaps in the biochemical network. These included the following: (i) vitamin transport systems such as nicotinic acid uptake, (ii) specific *S. thermophilus* protein synthesis based on experimental data, and (iii) different uptake systems such as oxygen diffusion, a proton symporter for lactate. The current model consists of 429 genes (23% of the total number of genes) and 522 model reactions, 79 (15%) of which are non-gene associated. Moreover, the biomass composition of this strain was determined in this study and compared with two other LAB (Table 1). The closely related strains *L. lactis* and *S. thermophilus* have comparable amounts of protein. Organic compounds in fermentation samples were measured by HPLC, on the basis of which fluxes were calculated (30). Both biomass data and fluxes were used for in silico simulations. The model of *S. thermophilus* is now at a stage where in silico growth can be simulated under different conditions.

Amino acid omissions. Experiments with single amino acid omissions in *S. thermophilus* have shown that the number and type of essential amino acids is strain dependent (9, 15, 17). In general, *S. thermophilus* has a much lower degree of auxotrophy for amino acids than other LAB (4), showing no growth only in the absence of histidine and clearly reduced growth in the absence of cysteine (see Table S1 in the supplemental material).

Multiple omissions of amino acids, performed in our laboratory, showed that *S. thermophilus* LMG18311 needs only histidine and one of the sulfur-containing amino acids (cysteine or methionine) in the presence of citrate for (minimal) growth (Table 2). We have performed the growth experiments on a minimal defined medium with histidine, cysteine, and glutamic acid, since the addition of glutamic acid improved the growth rate significantly and growth experiments showed that cysteine is preferred over methionine.

In silico predictions of the amino acid biosynthesis pathways of *S. thermophilus* LMG18311 were performed (11), and this strain indeed seems to contain all the genes coding for the enzymes required for the biosynthesis of all amino acids except histidine. This analysis also showed that *yhcE* is truncated by a conserved stop codon. The product of *yhcE* shows similarity to the vitamin B_{12}-independent 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase. Its orthologue in *L. lactis* is involved in the synthesis of cysteine from methionine. This gene inactivation may explain the auxotrophy for one of the two sulfur amino acids. Even though the genome of LMG18311 lacks a glutamate synthase gene, the strain shows (minimal) growth in the presence of citrate when both glutamate and glutamine were depleted from the medium. How-

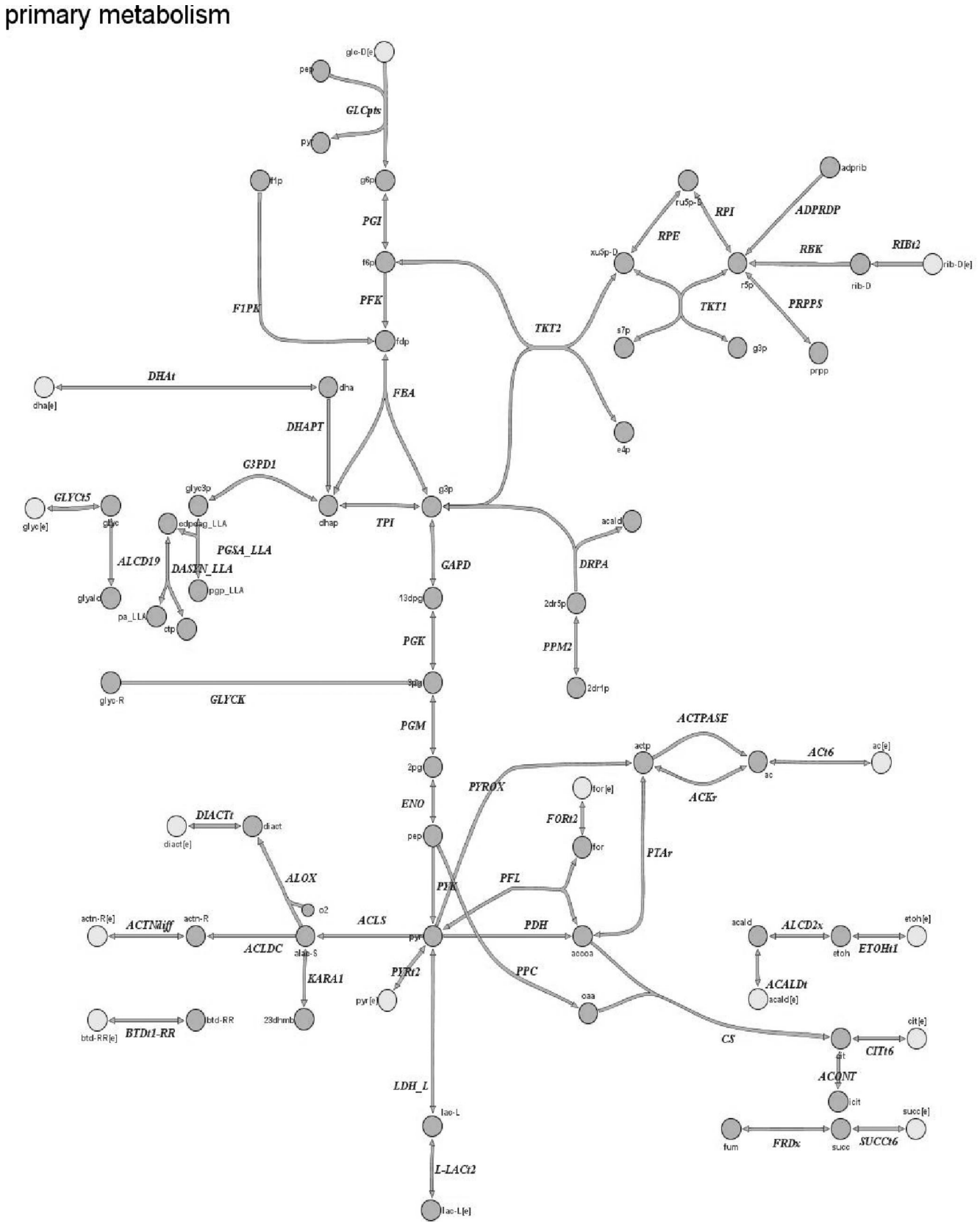

FIG. 1. Primary metabolism of *Streptococcus thermophilus*. Part of the total genome-scale metabolic model developed for *S. thermophilus*. Large bold capital italic letters indicate the enzymes, and smaller lowercase roman letters indicate the metabolites. For the complete model, see Fig. S1 in the supplemental material.

TABLE 1. Biomass composition of three different LAB

Compound	Overall biomass composition (%, wt/wt)		
	L. lactis (20)[a]	*L. plantarum* (30)	*S. thermophilus* (this study)[b]
Proteins	46	29.9	43.4
Lipids	3.4	6.3	6.1
Polysaccharides	12	9.9	24.1
DNA	2.3	1.9	1
RNA	10.7	9	8.2
Other	25.6	43	17.2

[a] A reference or source is provided in parentheses for each organism.
[b] Average for three fermentations.

TABLE 3. Essential amino acids for three different LAB

Strain (source or reference)	Essential amino acids
L. lactis MG1363 (12)	Glutamate, histidine, isoleucine, leucine, methionine, valine
L. plantarum WCFS1 (30)	Arginine, cysteine, glutamate, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine
S. thermophilus LMG18311 (Table 2 and this study)	Cysteine, histidine

ever, *S. thermophilus* possesses a pathway for the synthesis of glutamate from citrate via 2-oxoglutarate involving glutamate dehydrogenase and glutamine synthetase for interconversion between glutamic acid and glutamine.

Different LAB have different absolute requirements for amino acids; *S. thermophilus* only needs 2 amino acids, as described above, whereas *L. lactis* and *L. plantarum* need 6 and 11 amino acids for minimal growth, respectively (12, 30) (Table 3).

GC analyses. In order to get an overview of flavor formation by the three different LAB, we compared fermentation samples using GC. The headspaces of steady-state samples of *S. thermophilus* LMG18311, *L. lactis* MG1363, and *L. plantarum* WCFS1 grown on CDM (containing all amino acids) were compared. The metabolic activities of the fermenting microbes (22) were investigated through flavor profiles in the fermentation fluids corrected for the medium components at the start of the experiments. An overview of the volatile metabolic products is shown in Fig. 2, 3, and 4, and they show multiple differences in the volatile profiles of different strains. Many volatiles or flavors are produced during amino acid metabolism. When the results of the GC analyses of the three LAB are compared, they show that *S. thermophilus* is able to produce a broad variety of flavors. In combination with the low requirements of amino acids (only two), this reflects a relatively complete set of amino acid biosynthetic and amino acid-converting pathways. When *S. thermophilus* grows on CDM, all amino acids are consumed in small amounts (data not shown). *L. lactis* and *L. plantarum* need more amino acids (6 and 11, respectively) for minimal growth, and especially *L. plantarum* produces less flavors.

TABLE 2. Growth of *S. thermophilus* after 24 h under multiple amino acid omissions[a]

Amino acid composition in CDM (g/liter)	OD_{600}
All amino acids	1.55
No amino acids	0[b]
Only His (0.15) and Cys (0.39)	0.6[c]
Only His, Cys, Glu (0.4)	0.69
Only His, Cys, Glu, Phe (0.28)	0.75
Only His, Cys, Glu, Ser (0.34)	0.83
Only His, Cys, Glu, Ala (0.24)	0.44
Only His, Cys, Glu, Val (0.33)	0.72
Only His, Cys, Glu, Phe, Ser	0.73
Only His, Cys, Glu, Phe, Ala	0.53
Only His, Cys, Glu, Phe, Val	0.61
Only His, Cys, Glu, Ser, Ala	0.69
Only His, Cys, Glu, Ala, Val	0.82

[a] Data shown are the averages of three parallel cultures. Additional data for these amino acid omission experiments are shown (see Table S1 in the supplemental material).
[b] Negative control (should be 0).
[c] Growth after 48 h.

One of the identified compounds produced by all three LAB is acetaldehyde. As described previously (5), *S. thermophilus* can convert threonine into acetaldehyde and glycine by threonine aldolase activity. *L. lactis* and *L. plantarum*, among others, can produce acetaldehyde during lactose metabolism by pyruvate decarboxylation (3). This difference in pathways leading to the same compound can also be visualized in the SimPheny models, as was shown in our previous paper (22).

Homofermentative metabolism. *S. thermophilus* was grown under chemostat conditions on CDM containing all amino acids. Steady-state fermentation samples (dilution rate, 0.1 to 0.4 h^{-1}) of *S. thermophilus* were used for different analyses. The supernatant of these samples was analyzed by using HPLC and was compared with the composition of the growth medium to determine which compounds are produced and consumed during growth (Table 4).

The HPLC analysis shows that *S. thermophilus* consumes all the glucose and some of the citric acid. *S. thermophilus* produces mainly lactate, and only small amounts of pyruvate, succinate, and formate are formed. The model strongly suggests that homofermentative lactic acid production is the only primary metabolism operating in *S. thermophilus*, and this is confirmed by our fermentation data and also by others (11). The mixed acid fermentation (acetate, formate, and ethanol) is metabolically the most efficient route for LAB, whereas the homolactic route is catalytically more efficient (10). Both *L. lactis* and *L. plantarum* can grow via homolactic (high dilution rates) or mixed-acid (low dilution rates) fermentation (10, 30). Because *S. thermophilus* has pseudogenes in the primary metabolism that prevent the formation of ethanol, acetate formation will cause a redox problem, and hence, the only possible route is the homolactic fermentation at both high and low dilution rates.

Flux balance analysis (FBA) was carried out within the SimPheny software (30). FBA is an optimization technique that can be used as a tool to predict the metabolic possibilities given mass balance and capacity constraints (24). FBA correctly pre-

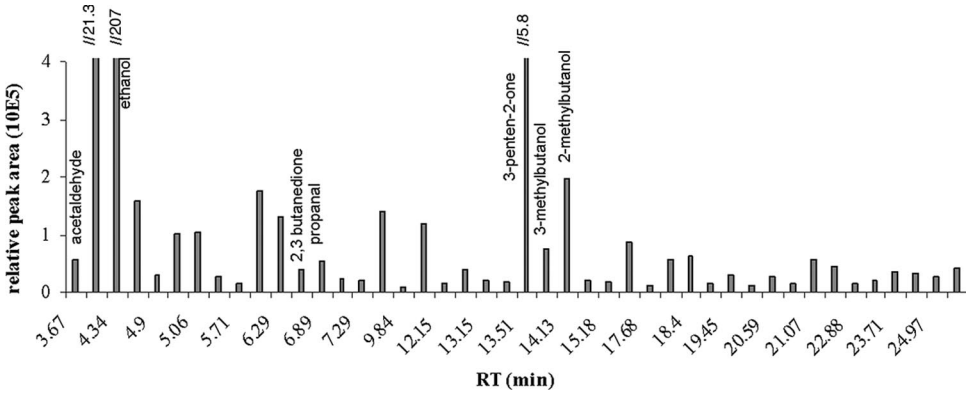

FIG. 2. Major volatiles formed during growth by *L. lactis* on CDM. Relative peak areas are expressed as arbitrary units, and the areas of three peaks are indicated since they are beyond the scale. Some important peaks are indicated. For all the identified metabolites for *L. lactis*, see Table S2 in the supplemental material.

dicted homolactic fermentation in *S. thermophilus*, in contrast to what was found for *L. plantarum* (30) and *L. lactis* (20). Based on the sequenced genome of strain LMG18311 that was visualized on the model, it is known that this strain does not have the oxidative part of the pentose phosphate pathway (PPP). The absence of a complete PPP may have important consequences for the redox balance and thereby may potentially influence primary metabolism.

DISCUSSION

In this paper, a comparative analysis of three LAB, *S. thermophilus*, *L. lactis*, and *L. plantarum*, is described. Comparative analysis can provide extra insight into metabolism, such as flavor formation and growth rate, and it can also reveal the absence of an important pathway in one of the strains because it is present in the other strains and vice versa. An illustrative example of this is the extensive flavor-forming potential of *S. thermophilus*. This was noticed only because we analyzed different strains simultaneously. Useful tools to compare different organisms are genome-scale metabolic models. Complete models are available for *L. lactis* and *L. plantarum*, and in this paper, we describe the construction of such a genome-scale model for *S. thermophilus* LMG18311. These genome-scale models are of course never complete and can always be expanded with new insights. Growth can be simulated under different conditions with these models. With some given constraints, such as lactose excess or different pH values, growth can be predicted and can give insight into optimal growth conditions.

The most obvious difference between the three bacteria, and therefore also the models, is the size of the genome and thus the number of genes. The model of *L. plantarum* contains 3,064 genes compared to 2,563 genes in the *L. lactis* model and 1,889 genes (or gene fragments) in the *S. thermophilus* model. This would suggest more extensive metabolisms for *L. plantarum* and *L. lactis*. But the total absolute numbers of reactions in the three models are closely similar: 522 for *S. thermophilus*, 598 for *L. plantarum*, and 598 for *L. lactis.* Based on the amino acid requirements and flavor analyses as described in Results, it seems that *S. thermophilus* has a more extensive amino acid metabolism than the other two LAB. *S. thermophilus* needs only two amino acids, histidine and cysteine, for minimal growth, it can degrade all amino acids, and it is able to produce varied amounts of amino acid-derived flavors. The genome-scale model, supported by the overall experimental data, suggests a rather complete set of amino acid biosynthesis pathways in *S. thermophilus*. This is unexpected, because *S. thermophilus* has been used for centuries for the production of yogurt. The

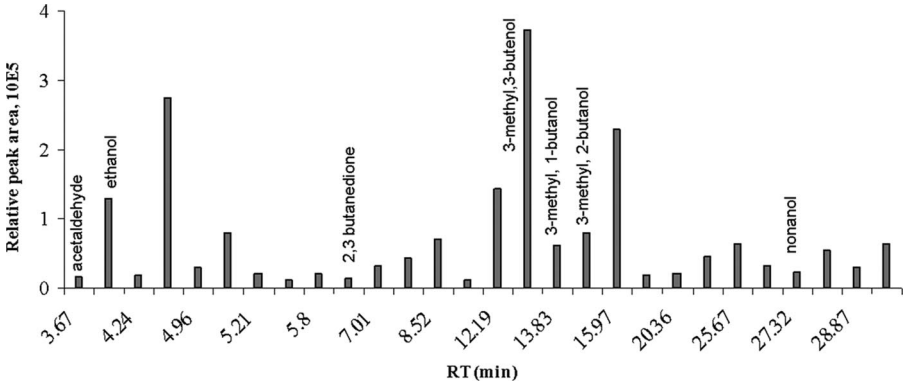

FIG. 3. Major volatiles formed during growth by *L. plantarum* on CDM. Relative peak areas are expressed as arbitrary units. Some important peaks are indicated. For all the identified metabolites for *L. plantarum*, see Table S3 in the supplemental material.

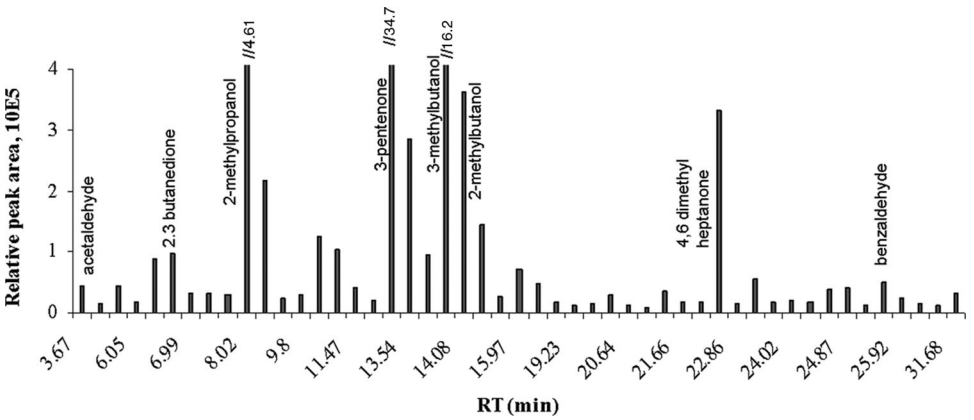

FIG. 4. Major volatiles formed during growth by *S. thermophilus* on CDM. Relative peak areas are expressed as arbitrary units, and the areas of three peaks are indicated since they are beyond the scale. Some important peaks are indicated. For all the identified metabolites for *S. thermophilus*, see Table S4 in the supplemental material.

LMG18311 strain is also a yogurt strain (11). The assumption is that *S. thermophilus* has evolved in this protein-rich environment (milk), and therefore, one might expect the loss of one or more amino acid biosynthesis pathways, but this is clearly not the case. It would be interesting to see if all these pathways operate under all conditions during the different dairy fermentation processes. These studies, in which expression data under different interesting conditions are involved, are currently under investigation. Intriguingly, *Lactobacillus delbrueckii* subsp. *bulgaricus*, an organism that is most often cocultivated with *S. thermophilus* for yogurt manufacturing, did follow this expected path and lost most of its amino acid biosynthetic capacity (31). An explanation for this unexpected behavior of *S. thermophilus* can be that amino acid metabolism not only is important for the synthesis of amino acids but also plays a role in maintaining the redox balance. Another explanation can be that *S. thermophilus* strains are selected for quick growth and acidification in milk, as available amino acids are rate limiting in milk. To support such quick growth, maintenance of nearly all amino acid pathways is required.

In Results, an in silico prediction of the amino acid biosynthesis pathways is described. This analysis showed that *ychE* is truncated by a conserved stop codon. It would be interesting to reconstitute this codon and study the effect of an activated codon. This mutated strain probably needs only one amino acid (histidine), and complete pathways for the sulfur amino acid metabolism may have important effects on the flavor formation.

TABLE 4. HPLC analyses of fermentation cell supernatants[a]

Growth medium	Compound concn (mM)[b]					
	Citric acid	Pyruvate	Lactic acid	Formic acid	Acetic acid	Glucose
CDM undiluted	2.49	ND	ND	ND	12.11	25.46
CDM dilutions						
$D = 0.1$	1.41	ND	20.41	ND	9.90	0.09
$D = 0.2$	1.39	ND	30.55	0.84	9.56	0.21
$D = 0.3$	1.70	0.12	33.08	1.28	10.83	ND
$D = 0.4$	1.99	0.21	34.70	1.83	12.01	0.36

[a] *S. thermophilus* was grown under chemostat conditions at a dilution rate (D) of 0.1 h^{-1} to 0.4 h^{-1} on CDM (5 g $liter^{-1}$ glucose) containing all amino acids. The table shows steady-state concentrations of the various metabolites formed or utilized in millimolar concentrations.

[b] Averages of two duplicates. ND, not detected.

A result from our experimental data, those described in the literature (11), and a prediction of the genome-scale model is that *S. thermophilus* has a simple primary metabolism because the number of pyruvate branches is limited, especially those which are important for NAD^+ regeneration for glycolysis, as there is no real alternative to lactate dehydrogenase for NAD^+ regeneration. Due to this, there is really only one possible route, leading to an equilibrated redox balance for glucose catabolism when *S. thermophilus* grows anaerobically, and that is the homolactic route. Therefore, FBA does predict the right growth rate and product formation rates in *S. thermophilus*. In *L. plantarum* and *L. lactis*, FBA invariably predicts the use of an alternative pathway with a higher ATP yield (mixed-acid fermentation), and homolactic fermentation cannot be predicted by FBA.

Another striking difference between *S. thermophilus*, *L. lactis*, and *L. plantarum* is the absence of a complete PPP. Three genes coding for the enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, and phosphogluconate dehydrogenase are missing; these three enzymes form the oxidative part of the PPP. This might have important consequences for the NADPH generation, the ribonucleotides, and aromatic amino acid synthesis. There might be a link between the simple primary metabolism (limited number of pyruvate branches and the absence of a complete PPP) and the complex amino acid metabolism via redox constraints, a hypothesis that is currently under investigation.

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